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Irreversible inactivation of snake venom L-amino acid oxidase by covalent modification during catalysis of L-propargylglycine

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ABSTRACT

Snake venom L-amino acid oxidase (SV-LAAO, a flavor-enzyme) has attracted considerable attention due to its multifunctional nature, which is manifest in diverse clinical and biological effects such as inhibition of platelet aggregation, induction of cell apoptosis and cytotoxicity against various cells. The majority of these effects are mediated by H_2O_2 generated during the catalytic conversion of L-amino acids. The substrate analog L-propargylglycine (LPG) irreversibly inhibited the enzyme from *Crotalus adamanteus* and *Crotalus atrox* in a dose- and time-dependent manner. Inactivation was irreversible which was significantly protected by the substrate L-phenylalanine. A Kitz–Wilson replot of the inhibition kinetics suggested formation of reversible enzyme–LPG complex, which occurred prior to modification and inactivation of the enzyme. UV–visible and fluorescence spectra of the enzyme and the cofactor strongly suggested formation of covalent adduct between LPG and an active site residue of the enzyme. A molecular modeling study revealed that the FAD-binding, substrate-binding and the helical domains are conserved in SV-LAAOs and both His223 and Arg322 are the important active site residues that are likely to get modified by LPG. Chymotrypsin digest of the LPG inactivated enzyme followed by RP-HPLC and MALDI mass analysis identified His223 as the site of modification. The findings reported here contribute towards complete inactivation of SV-LAAO as a part of snake envenomation management.

1. Introduction

L-amino acid oxidase (LAAO), a dimeric enzyme containing non-covalently bound FAD as cofactor, is widely present in eukaryotic and prokaryotic organisms [1]. They catalyse stereospecific oxidative deamination of L-amino acids to α-keto acid via α-imino acid intermediate along with generation of ammonia and hydrogen peroxide (Fig. 1). These enzymes exhibit a marked preference for hydrophobic amino acids like phenylalanine, tryptophan, tyrosine and leucine as substrate [2,3]. This substrate preference originates from the binding of hydrophobic side chains of amino acids with the enzyme. The catalysis is proposed to follow either of the two different mechanisms: a carbanion pathway in which the proton is transferred to the FMN/FAD cofactor from the α-carbon atom of the substrate leaving a negative charge followed by two electron transfer. Alternately, a hydride transfer pathway in which an alpha hydrogen atom is transferred as a hydride ion carrying two electrons simultaneously. Recent structural and mechanistic studies on D-amino acid oxidase (DAAO) support the hydride transfer mechanism [4].

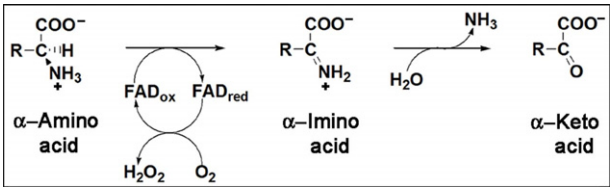

Fig. 1. Catalytic oxidation of L-amino acid oxidase. The first half reaction involves the conversion of FAD to $FADH_2$ with concomitant oxidation of the amino acid to an imino acid. Another oxidative half reaction completes the catalytic cycle by reoxidizing the $FADH_2$ with oxygen, producing hydrogen peroxide. The unstable imino acid is then hydrolyzed by the enzyme bound water to form the final product keto acid.

LAAOs isolated from snake venoms are the best characterized members of this enzyme family. High abundance of the enzyme in

Abbreviations: CHD, 1,2-cyclohexanedione; DEPC, diethylpyrocarbonate; FAD, flavin adenine dinucleotide; Gdn-HCl, guanidine hydrochloride; LAAO, L-amino acid oxidase (EC. 1.4.3.2); LPG, L-propargylglycine; L-Phe, L-phenylalaine; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; TNBS, trinitrobenzene sulfonic acid.

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snake venom causes potential toxicity, as it induces impairment of platelet aggregation together with necrotic and apoptotic cell death [2,5,6]. This effect is primarily attributed to the production of high concentration of localized H_2O_2 [7,8]. LAAO from *Crotalus adamanteus* and *Crotalus atrox* can associate specifically with mammalian endothelial cells possibly through the glycosylation site of the enzyme [7,9]. Such association and interaction of LAAO with bacterial cell was observed in case of *Trichoderma harzianum ETS323* [10]. It is predicted that the glycan moiety of LAAO is used in docking the enzyme to the cell surface, thus enhancing the localization of H_2O_2 [7,9]. This enzyme share sequence similarity with human monoamine oxidase (MAO) and may involved in allergic inflammatory response [11]. Recent crystallographic studies of LAAO from *Calloseasma rhodostoma* complexed with L-Phe and *o*-aminobenzoic acid reveal that the catalytic site of each subunit of the dimeric enzyme is composed of three parts: a FAD-binding domain, a substrate-binding domain and a helical domain [12,13]. High degree of structural similarity of these three domains of LAAO from *C. rhodostoma, Bothrops jararacussu* and *Bothrops moojeni* was observed through comparative sequence homology and molecular modeling [14]. Also LAAO from *C. rhodostoma* shares 83% sequence identity with that of both *C. adamanteus* and *C. atrox*. Overall, snake venom LAAOs are highly conserved with respect to their structure, function and substrate specificity.

A variety of mechanism based inhibitors have been reported for a number of flavoenzymes [15,16]. These inhibitors can inactivate the enzymes either by modifying the cofactor or a reactive amino acid residue during catalytic turnover [17–24]. Success of such inhibitors has generated an increasing interest towards designing of suicidal substrate for LAAO for partial neutralization of venom toxicity. Earlier reports indicate that L-propargylglycine (LPG) acts as a substrate of lower affinity and not an inhibitor of LAAO from *C. adamanteus* whereas D-propargylglycine acts as an inhibitor of D-amino acid oxidase (DAAO) [25–27]. Inactivation of LAAO by LPG was not realized, either due to the low concentration of LPG applied or the kinetic of inactivation was followed for a short time. Here, we report that LPG irreversibly inactivates LAAO by modifying active site His223 during its catalytic turnover. Thus it acts as a mechanism-based inhibitor. Similar results were also observed with LAAO from *C. atrox* venom.

The working hypothesis of the current observation is that there are multiple substrate/inhibitor binding sites in the catalytic funnel of LAAO of which one is responsible for catalytic turnover. It is believed that LPG at low concentration binds exclusively at the catalytic site and undergoes enzymatic conversion. But at higher concentration, LPG binds at multiple sites including the catalytic site of LAAO. Binding of LPG at noncatalytic sites probably induces conformation change of the enzyme that leads to covalent modification of the enzyme with LPG undergoing conversion at the catalytic site leading to a dead end complex. This assumption is based on the crystal structures of LAAOs where existence of multiple substrate/inhibitor binding sites in the catalytic funnel is evident [12,13]. The purpose of this study is to gain insight for designing specific and potent inhibitors of LAAO.

2. Results

2.1. Oxidation of LPG by LAAO

Unless mentioned otherwise, LAAO used in this work refers to the enzyme from *C. adamanteus*. LPG, a propargyl derivative of L-glycine has all the features that are essential for becoming a substrate of LAAO, e.g., amino and carboxylic groups along with a hydrophobic side chain. LPG up to 500 μM acts as a substrate of LAAO for at least 180 min when monitored at 320 nm (Fig. 2A). The initial rates and the amounts of product formed by180 min when it is assumed that the reaction is complete, show linear dependency with LPG concentration (Fig. 2B and C). Since no inactivation was observed during the course of turnover, it is clear that irreversible enzyme–inhibitor adduct is not formed under that concentration of LPG. Therefore, it is assumed that the absorbance at 320 nm is exclusively contributed by the LPG oxidation product 3-amino-5-methylene-5*H*-furan-2-one and not by the adduct that also absorbs at 320 nm (discussed later).

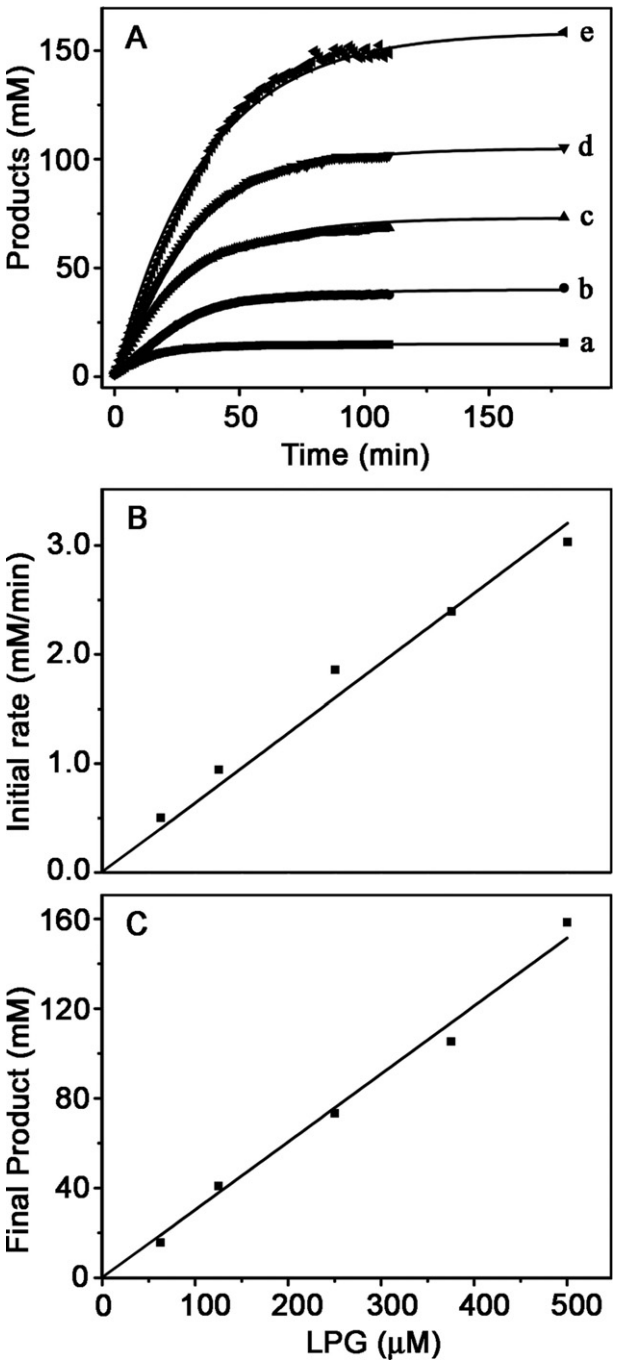

Fig. 2. (A) Time course of oxidation of (a) 62.5, (b) 125, (c) 250, (d) 375 and (e) 500 μM of LPG by 81.22 nM of LAAO. Experimental conditions have been mentioned in the text. (B) Linear dependence of the initial rate and (C) product formation at 180 min, when the reactions was assumed to be complete with the concentration of LPG. R^2 (regression coefficient) values are 0.992 and 0.996, respectively.

In order to compare the affinity of the substrates L-Phe and LPG, Michaelis–Menten plots were constructed. While measuring the initial rates, the reactions were followed for 120 s where linear increment of product formation was observed. Dependence of initial